

Plants expressing  $\Delta 6$ -desaturase genes, PUFAS-containing [sic] oils from these plants, and a process for the preparation of unsaturated fatty acids

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The present invention relates to an improved process for the preparation of unsaturated fatty acids and to a process for the preparation of triglycerides with an increased content of unsaturated fatty acids. The invention relates to the generation 10 of a transgenic organism, preferably of a transgenic plant or of a transgenic microorganism, with an increased content of fatty acids, oils or lipids with  $\Delta 6$  double bonds owing to the expression of a moss  $\Delta 6$ -desaturase [sic].

15 The invention furthermore relates to transgenic organisms containing a  $\Delta 6$ -desaturase gene, and to the use of the unsaturated fatty acids or triglycerides with an increased content of unsaturated fatty acids which have been prepared by the process.

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Fatty acids and triglycerides have a multiplicity of uses in the food industry, in livestock nutrition, in cosmetics and in the pharmaceutical sector. They are suitable for a wide variety of uses depending on whether they are free saturated or unsaturated 25 fatty acids or triglycerides with an increased content of saturated or unsaturated fatty acids; thus, for example, polyunsaturated fatty acids are added to baby food to increase the nutritional value. The various fatty acids and triglycerides are obtained mainly from microorganisms such as *Mortierella* or 30 from oil-producing plants such as soybean, oilseed rape, sunflower and others, usually resulting in the form of their triacyl glycerides. However, they can also be obtained from animal species such as fish. The free fatty acids are advantageously prepared by saponification.

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Depending on the intended use, oils with saturated or unsaturated fatty acids are preferred; thus, for example, lipids with unsaturated fatty acids, specifically polyunsaturated fatty acids, are preferred in human nutrition because they have a 40 beneficial effect on the blood cholesterol level and thus on the possibility of heart disease. A positive action on carcinogenesis is also attributed to the unsaturated fatty acids. Moreover, they are important starting materials for the synthesis of compounds which govern important biological processes within the organism. 45 They are therefore used in various dietetic foodstuffs or medicaments.

Owing to their beneficial properties, there has been no lack of attempts in the past to make available genes which are involved in the synthesis of fatty acids or triglycerides for the production of oils in various organisms with a modified content of unsaturated fatty acids. Thus, a  $\Delta 9$ -desaturase is described in WO 91/13972 and its US equivalent. WO 93/11245 claims a  $\Delta 15$ -desaturase, while WO 94/11516 claims a  $\Delta 12$ -desaturase.  $\Delta 6$ -desaturases are described in Girke et al. (The Plant Journal, 15, 1998: 39-48), Napier et al. (Biochem. J., 330, 1998: 10 611-614), Murata et al. (Biosynthesis of  $\gamma$ -linolenic acid in cyanobacterium *Spirulina patensis*, pp. 22-32, In:  $\gamma$ -linolenic acid, metabolism and its roles in nutrition and medicine, Huang, Y. and Milles, D.E. [eds.], AOC Press, Champaign, Illinois), Sayanova et al. (Proc. Natl. Acad. Sci. USA, 94, 1997: 15 4211-4216), WO 98/46764, Cho et al. (J. Biol. Chem., 274, 1999: 471-477), Aki et al. (Biochem. Biophys. Res. Commun., 255, 1999: 575-579), and Reddy et al. (Plant Mol. Biol., 27, 1993: 293-300). Further desaturases are described, for example, in EP-A-0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, 20 WO 95/18222, EP-A-0 794 250, Stukey et al., J. Biol. Chem., 265, 1990: 20144-20149, Wada et al., Nature 347, 1990: 200-203 or Huang et al., Lipids 34, 1999: 649-659. Further  $\Delta 6$ -desaturase are described in WO 93/06712, US 5,614,393, US5,614,393, WO 96/21022, WO 00/21557 and WO 99/27111. The biochemical 25 characterization of the various desaturases is, however, inadequate as yet because the enzymes, being membrane-bound proteins, can be isolated and characterized only with great difficulty (McKeon et al., Methods in Enzymol. 71, 1981: 12141-12147, Wang et al., Plant Physiol. Biochem., 26, 1988: 30 777-792). As a rule, membrane-bound desaturases are characterized by introducing them into a suitable organism which is subsequently tested for enzyme activity by analyzing the starting material and the product. The use for production in transgenic organisms described as in WO 98/46763 WO 98/46764, WO 98/46765 35 [sic]. The expression of various desaturases as in WO 99/64616 or WO 98/46776 and the formation of polyunsaturated fatty acids is also described and claimed here. As regards the expression efficacy of desaturases and their effect on the formation of polyunsaturated fatty acids, it must be noted that expression of 40 an individual desaturase as described in the above prior art only led to, and leads to, low contents of unsaturated fatty acids, for example  $\Delta 6$ -unsaturated [sic] fatty acids/lipids such as, for example,  $\gamma$ -linoleic acid, being achieved.

45 There is thus still a great need for novel genes which are better suited and which encode enzymes which are involved in the biosynthesis of unsaturated fatty acids and which allow them to

be produced on an industrial scale. Furthermore, there is still a need for improved methods of obtaining the highest possible contents of polyunsaturated fatty acids.

5 It is an object of the present invention to provide a process for the preparation of unsaturated fatty acids using genes which encode, for example, desaturase enzymes and which are involved in the synthesis of polyunsaturated fatty acids in the seeds of an oil crop, thus increasing the content of polyunsaturated fatty acids.

We have found that this object is achieved by a process for the preparation of unsaturated fatty acids, which comprises introducing, into an organism, at least one isolated nucleic acid sequence encoding a polypeptide having  $\Delta 6$ -desaturase activity, selected from the group consisting of:

- a) a nucleic acid sequence having the sequence shown in SEQ ID NO: 1,
  - 20 b) nucleic acid sequences which, as a result of the degeneracy of the genetic code, are derived from the [lacuna] in SEQ ID NO: 1,
  - c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1 which encode polypeptides with the amino acid sequences shown in SEQ ID NO: 2 and have at least 50% homology at the amino acid level without substantially reducing the enzymatic action of the polypeptides,
- 30 and culturing this organism, where the cultured organism contains at least 1 mol% of unsaturated fatty acids based on the total fatty acid content in the organism.

Culturing this organism means both growing plants and culturing eukaryotic or prokaryotic microorganisms such as bacteria, yeasts, fungi, ciliates, algae, cyanobacteria, animal or plant cells or cell associations, or rearing animals.

The organisms obtained by the processes according to the invention contain, as a rule, unsaturated fatty acids in the form of bound fatty acids, i.e. the unsaturated fatty acids exist predominantly in the form of their mono-, di- or triglycerides, glycolipids, lipoproteins or phospholipids such as oils or lipids or else as fatty acids bound as esters or amides. Free fatty acids are also present in the organisms in the form of the free fatty acids or in the form of their salts. Advantageously, the free or bound unsaturated fatty acids have an increased content

of fatty acids with  $\Delta 6$  double bonds, such as, advantageously,  $\gamma$ -linoleic acid, which is increased over that of the starting organisms. The organisms obtained by culturing in the process according to the invention, and the unsaturated fatty acids which 5 they contain, can be used directly, for example for the production of pharmaceutical products, of agrochemicals, feeds or foodstuffs or else after isolation from the organisms. All steps of the purification of the unsaturated fatty acids can be used, that is to say that [lacuna] from crude extracts of the fatty 10 acids up to fully purified fatty acids are suitable for preparing the abovementioned products. In an advantageous embodiment, the bound fatty acids can be liberated from the, for example, oils or lipids for example by hydrolysis with bases, such as, for example, with NaOH or KOH. These free fatty acids can be used 15 directly in the mixture obtained or after further purification for producing pharmaceutical products, agrochemicals, feeds of foodstuffs. Also, the bound or free fatty acids can be used for transesterification or esterification, for example with other mono, di- or triglycerides or glycerol in order to increase the 20 content of unsaturated fatty acids in these compounds, for example in the triglycerides.

The invention furthermore relates to a process for the preparation of triglycerides with an increased content of 25 unsaturated fatty acids by incubating triglycerides with saturated or unsaturated or saturated and unsaturated fatty acids with at least one of the proteins encoded by the sequence SEQ ID NO: 2. The processes are advantageously carried out in the presence of compounds which are capable of accepting or donating 30 reduction equivalents. The fatty acids can subsequently be released from the triglycerides.

The abovementioned methods advantageously allow fatty acids of bound fatty acids such as triglycerides with an increased content 35 of fatty acids with  $\Delta 6$  double bonds to be synthesized.

Organisms which may be mentioned for the abovementioned processes are, for example, plants such as *Arabidopsis*, barley, wheat, rye, oats, maize, soybean, rice, cotton, sugarbeet, tea, carrot, 40 *capsicum*, canola, sunflower, flax, hemp, potato, triticale, tobacco, tomato, oilseed rape, coffee, tapioca, carcabá, arrowroot, tagetes, alfalfa, peanut, castor, coconut, oilpalm, safflower (*Carthamus tinctorius*), lettuce and the various tree, nut and grapevine species, or cacao bean, microorganisms such as 45 the fungi *Mortierella*, *Saprolegnia* or *Pythium*, bacteria such as the genus *Escherichia*, cyanobacteria, algae or protozoans such as dinoflagellates such as *Cryptocodium*. Preferred organisms are

those which are naturally capable of synthesizing substantial amounts of oils, such as microorganisms such as fungi such as Mortierella alpina, Pythium insidiosum or plant such as soybean, oilseed rape, coconut, oil palm, canola, safflower (Carthamus tinctorius), castor, calendula, linseed, borage, peanut, cacao bean or sunflower, with soybean, oilseed rape or sunflower being especially preferred.

Depending on the host organism, the organisms used in the processes are cultured or grown in the manner known to the skilled worker. Microorganisms, such as bacteria, fungi, ciliates, plant or animal cells, are usually cultured in a liquid medium which contains a carbon source, in most cases in the form of sugars, a nitrogen source, in most cases in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as iron salts, manganese salts and magnesium salts and, if appropriate, vitamins, at temperatures between 0°C and 100°C, preferably between 10°C and 60°C, either with passing in of oxygen or in the absence of oxygen, depending on the organism. It is possible in this context to maintain the pH of the medium at a fixed value, that is to say the pH is regulated during culturing or else the pH is not regulated and changes during culture. Culturing can be carried out batchwise, semi-batchwise or continuously. Nutrients can be introduced at the beginning of the fermentation or subsequently fed semicontinuously or continuously. A culture on solid media is also possible.

After the transformation, plants are, as a rule, first regenerated and then cultured or grown as customary. This can be done in the greenhouse or in the open.

After cultivation, the lipids are obtained from the organisms in the customary manner. To this end, the organisms can first be disrupted after harvesting or else used directly. The lipids are advantageously extracted with suitable solvents such as apolar solvents such as hexane or ethanol, isopropanol or mixtures such as hexane/isopropanol, phenol/chloroform/isoamyl alcohol at temperatures between 0°C to 80°C, preferably between 20°C to 50°C. As a rule, the biomass is extracted with an excess of solvent, for example an excess of solvent to biomass of 1:4. The solvent is subsequently removed, for example via distillation. Extraction can also be effected by using supercritical CO<sub>2</sub>. After extraction, the residual biomass can be removed for example by filtration.

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The crude oil thus obtained can subsequently be purified further, for example by removing cloudiness by treating the oil with polar

solvents such as acetone or chloroform, followed by filtration or centrifugation. A further purification by chromatographic methods, distillation or crystallization is also possible.

- 5 To obtain the free fatty acids from the triglycerides, they are saponified in the customary manner as described above.

The invention furthermore relates to unsaturated fatty acids and to triglycerides [sic] with an increased content of unsaturated 10 fatty acids which have been prepared by the abovementioned methods, and to their use for the production of foodstuffs, feeds, cosmetics or pharmaceuticals. To this end, they are added in customary quantities to the foodstuffs, the feeds, the cosmetics or the pharmaceuticals.

- 15 In the process according to the expression, higher contents of unsaturated fatty acids such as  $\gamma$ -linolenic acid were obtained by expressing a moss  $\Delta 6$ -desaturase in organisms such as fungi, bacteria, animal or plants, preferably fungi, bacteria and 20 plants, especially preferably in plants, very especially preferably in oil crops such as oilseed rape, canola, linseed, soybean, sunflower, borage, castor, oilpalm, safflower (*Carthamus tinctorius*), coconut, peanut or cacao bean. Expression in field crops such as maize, wheat, rye, oats, triticale, rice, barley, 25 alfalfa or bush plants (coffee, cacao, tea) is also advantageous. Expression in the abovementioned organisms of a gene which encodes a moss  $\Delta 6$ -desaturase [sic] allows contents of 30 unsaturated fatty acids of at least 1 mol%, preferably at least 3 mol%, especially preferably at least 4 mol%, very especially preferably at least 5 mol%, to be achieved in the organisms.

Derivative(s) are to be understood as meaning, for example, functional homologues of the enzymes encoded by SEQ ID NO: 1 or their enzymatic activity, that is to say enzymes which catalyze 35 the same enzymatic reactions as those of SEQ ID NO: 1. These genes also make it possible advantageously to prepare unsaturated fatty acids with double bonds in position  $\Delta 6$ . Unsaturated fatty acids are to be understood hereinbelow as meaning doubly or polyunsaturated fatty acids which have double bonds. The double 40 bonds can be conjugated or unconjugated. The sequence stated in SEQ ID NO: 1 encodes an enzyme which has a  $\Delta 6$ -desaturase activity.

The enzyme  $\Delta 6$ -desaturase according to the invention 45 advantageously introduces a *cis* double bond in position C<sub>6</sub>-C<sub>7</sub> into fatty acid residues of glycerolipids (see SEQ ID NO: 1). Moreover, the enzyme has a  $\Delta 6$ -desaturase activity which

advantageously introduces exclusively a *cis* double bond in position C<sub>6</sub>-C<sub>7</sub> into fatty acid residues of glycerolipids. The enzyme with the sequence stated in SEQ ID NO: 1 also has this activity, which is that of a monofunctional Δ6-desaturase.

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The nucleic acids sequence(s) (the singular is intended to encompass the plural, and vice versa, for the application) or fragments thereof used in the process according to the invention can be used advantageously for isolating further genomic

10 sequences via homology screening.

The derivatives mentioned can be isolated, for example, from other organisms [lacuna] eukaryotic organisms such as plants, such as, especially, mosses, dinoflagellates or fungi.

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Derivatives or functional derivatives of the sequence stated in SEQ ID NO: 1 are furthermore to be understood as meaning, for example, allelic variants which have at least 50% homology at the deduced amino acid level, advantageously at least 70% homology,

20 preferably at least 80% homology, especially preferably at least 85% homology, and very especially preferably 90% homology. The homology was calculated over the entire amino acid region. The program PileUp, BESTFIT, GAP, TRANSLATE or BACKTRANSLATE (= constituent of the program package UWGCG, Wisconsin Package,

25 Version 10.0-UNIX, January 1999, Genetics Computer Group, Inc., Deverux et al., Nucleic. Acid Res., 12, 1984: 387-395) was used (J. Mol. Evolution., 25, 351-360, 1987, Higgins et al., CABIOS, 5 1989: 151-153). The amino acid sequences deduced from the specified nucleic acids can be found in sequence SEQ ID NO: 2.

30 Homology is to be understood as meaning identity, i.e. the amino acid sequences are at least 50% identical. The sequences according to the invention have at least 65%, preferably at least 70%, especially preferably 75%, very especially preferably at least 80%, homology at the nucleic acid level.

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Allelic variants comprise, in particular, functional variants which can be obtained from the sequence shown in SEQ ID NO: 1 by deletion, insertion or substitution of nucleotides, while retaining the enzymatic activity of the deduced synthesized

40 proteins.

Such DNA sequences can be isolated starting from the DNA sequence described in SEQ ID NO: 1 or parts of these sequences from other eukaryotes, such as, for example, those mentioned above, for

45 example using customary hybridization methods or the PCR technique. These DNA sequences hybridize with the abovementioned sequences under standard conditions. For hybridization, it is

advantageous to use short oligonucleotides, for example of conserved regions, which can be determined in a manner known to the skilled worker by comparisons with other desaturase genes. It is advantageous to use the histidine box sequences. However, it 5 is also possible to use longer fragments of the nucleic acids according to the invention or the complete sequences for the hybridization. These standard conditions vary depending on the nucleic acid used: oligonucleotide, longer fragment or complete sequence, or depending on which type of nucleic acid, DNA or RNA, 10 is used for the hybridization. Thus, for example, the melting temperatures for DNA:DNA hybrids are about 10°C lower than those for DNA:RNA hybrids of the same length.

Standard conditions mean, for example, depending on the nucleic 15 acid, temperatures between 42 and 58°C in an aqueous buffer solution with a concentration between 0.1 to [sic] 5 x SSC (1 x SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.2) or additionally in the presence of 50% formamide, such as, for example, 42°C in 5 x SSC, 50% formamide. The hybridization conditions for DNA:DNA 20 hybrids are advantageously 0.1 x SSC and temperatures between about 20°C to [sic] 45°C, preferably between about 30°C to [sic] 45°C. The hybridization conditions for DNA:RNA hybrids are advantageously 0.1 x SSC and temperatures between about 30°C to [sic] 55°C, preferably between about 45°C to [sic] 55°C. These 25 temperatures stated for the hybridization are melting temperatures calculated by way of example for a nucleic acid with a length of about 100 nucleotides and a G + C content of 50% in the absence of formamide. The experimental conditions for DNA hybridization are described in relevant textbooks of genetics 30 such as, for example, Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1989, and can be calculated by the formulae known to the skilled worker, for example depending on the length of the nucleic acids, the nature of the hybrids or the G + C content. Further information on hybridization can be found 35 by the skilled worker in the following textbooks: Ausubel et al. (eds), 1985, Current Protocols in Molecular Biology, John Wiley & Sons, New York; Hames and Higgins (eds), 1985, Nucleic Acids Hybridization: A Practical Approach, IRL Press at Oxford University Press, Oxford; Brown (ed), 1991, Essential Molecular 40 Biology: A Practical Approach, IRL Press at Oxford University Press, Oxford.

Derivatives are also to be understood as meaning homologues of the sequence SEQ ID No: 1, for example eukaryotic homologues, 45 truncated sequences, single-stranded DNA of the coding and noncoding DNA sequence or RNA of the coding and noncoding DNA

sequence.

Homologues of the sequence SEQ ID NO: 1 are furthermore to be understood as meaning derivatives such as, for example, promoter variants. These variants can be modified by one or more nucleotide exchanges, by insertion(s) and/or deletion(s), but without adversely affecting the functionality or efficacy of the promoters. Moreover, the promoters may have their efficacy increased by modification of their sequence, or be completely replaced by more effective promoters, even from heterologous organisms.

Derivatives are also advantageously understood as meaning variants whose nucleotide sequence in the region from -1 to -2000 in front of the start codon has been modified so that gene expression and/or protein expression is altered, preferably increased. Moreover, derivatives are also understood as meaning variants which have been modified at the 3' end.

The nucleic acid sequences encoding a Δ6-desaturase can be synthesized or obtained from nature or contain a mixture of synthetic or natural DNA constituents, or else be composed of various heterologous Δ6-desaturase gene sections from various organisms. In general, synthetic nucleotide sequences are produced using codons which are preferred by the host organisms in question, for example plants. As a rule, this leads to optimal expression of the heterologous genes. These codons which are preferred by plants may be determined from codons with the greatest protein frequency which are expressed in most plant species of interest. An example for *Corynebacterium glutamicum* is given in: Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Experiments of this type can be carried out by standard methods and are known to those skilled in the art.

Functionally equivalent sequences encoding the Δ6-desaturase gene are those derivatives of the sequence according to the invention which still have the desired functions, i.e. the enzymatic activity of the proteins, despite deviating nucleotide sequence. Functional equivalents thus encompass naturally occurring variants of the sequences described herein and artificial nucleotide sequences, for example obtained by chemical synthesis and adapted to the codon usage of a plant.

In addition, artificial DNA sequences are suitable as long as they confer, as described above, the desired property, for example the increase in the content of Δ6-double bonds in fatty acids, oils or lipids in the plant by overexpression of the

Δ6-desaturase gene in crop plants. Such artificial DNA sequences can be established for example, by backtranslation of proteins constructed by means of molecular modeling and having Δ6-desaturase activity, or by in-vitro selection. Techniques which are possible for the in-vitro evolution of DNA for modifying or improving the DNA sequences are described in Patten, P.A. et al., Current Opinion in Biotechnology 8, 724-733( 1997) or in Moore, J.C. et al., Journal of Molecular Biology 272, 336-347 (1997). Coding DNA sequences which have been obtained by backtranslating of a polypeptide sequence in accordance with the codon usage specific for the host plant are particularly suitable. This specific codon usage can be determined easily by a skilled worker familiar with methods of plant genetics by computer analysis of other, known genes of the plant to be transformed.

Further suitable equivalent nucleic acid sequences which must be mentioned are sequences which encode fusion proteins, where a Δ6-desaturase polypeptide or a functionally equivalent portion thereof is part of the fusion protein. The second portion of the fusion protein can be, for example, another polypeptide with enzymatic activity or an antigenic polypeptide sequence with the aid of which it is possible to detect Δ6-desaturase expression (for example myc-tag or his-tag). However, this preferably takes the form of a regulatory protein sequence such as, for example, an ER signal sequence, which guides the Δ6-desaturase protein to the desired site of action.

It is advantageously possible to combine the Δ6-desaturase gene in the process according to the invention with further genes of fatty acid biosynthesis. Examples of such genes are the acetyl transferases, further desaturases or elongases of unsaturated or saturated fatty acids as described in WO 00/12720. Advantageous for the in-vivo and, specifically, in-vitro synthesis is the combination with, for example, NADH cytochrome B5 reductases, which are able to accept or dominate reduction equivalents.

The proteins used in the process according to the invention are to be understood as meaning proteins which comprise an amino acid sequence shown in SEQ ID NO: 2 or a sequence which can be obtained therefrom by substitution, inversion, insertion or deletion of one or more amino acid residues, with the enzymatic activity of the protein shown in SEQ ID NO: 2 being retained or not substantially reduced. Not substantially reduced is to be understood as meaning all enzymes which still have at least 10%, preferably 20%, especially preferably 30%, of the enzymatic activity of the starting enzyme. It is moreover possible, for

example, to replace particular amino acids by those with similar physico-chemical properties (bulk, basicity, hydrophobicity and the like). For example, arginine residues are replaced by lysine residues, valine residues by isoleucine residues or aspartic acid residues by glutamic acid residues. However, it is also possible for one or more amino acids to be transposed in their sequence, added or deleted, or several of these measures can be combined with each other.

Derivatives are also to be understood as functional equivalents which comprise, in particular, also natural or artificial mutations of an originally isolated sequence encoding a  $\Delta 6$ -desaturase and which additionally show the required function, that is to say the enzymatic activity is not substantially reduced. Mutations encompass substitutions, additions, deletions, transpositions or insertions of one or more nucleotide residues. Thus, for example, the present invention also extends to those nucleotide sequences which are obtained by modification of the  $\Delta 6$ -desaturase nucleotide sequence. The aim of such a modification may be, for example, to localize further the coding sequence contained therein or, for example, also to insert further restriction enzyme cleavage sites.

Functional equivalents are also those variants whose function is, compared with the initial gene or gene fragment, attenuated (= not substantially reduced) or enhanced (= enzyme activity is greater than the activity of the initial enzyme, that is to say the activity is over 100%, preferably over 110%, particularly preferably over 130%).

The nucleic acid sequences mentioned above which can be used in the process according to the invention are advantageously inserted into an expression cassette in order to introduce them into a host organism. However, the nucleic acid sequences can also be introduced directly into the host organism. The nucleic acid sequence may advantageously be, for example, a DNA or cDNA sequence.

Coding sequences which are suitable for insertion into an expression cassette are, for example, those which encode a  $\Delta 6$ -desaturase with the above-described sequences and which impart, to the host, the ability of overproducing fatty acids, oils or lipids with double bonds in position  $\Delta 6$ . These sequences can be of homologous or heterologous origin.

An expression cassette (= nucleic acid construct or fragment) is to be understood as meaning the sequence stated in

SEQ ID NO: 1 which is the result of the genetic code and/or its functional or nonfunctional derivatives which have advantageously been linked functionally to one or more regulatory signals to increase gene expression and which control expression of the 5 coding sequence in the host cell. These regulatory sequences are intended to make specific expression of the genes and protein expression possible. This may mean, for example, depending on the host organism, that the gene is expressed and/or overexpressed only after induction, or that it is expressed and/or 10 overexpressed immediately. For example, these regulatory sequences are sequences to which inducers or repressors bind and thus regulate expression of the nucleic acid. In addition to these novel regulatory sequences or in place of these sequences, it is possible for the natural regulation of these sequences 15 still to be present in front of the actual structural genes and, where appropriate, to have been genetically modified so that natural regulation has been switched off and expression of the genes has been increased. However, the gene construct may also have a simple structure, that is to say no additional regulatory 20 signals have been inserted in front of the nucleic acid sequence or its derivatives and the natural promoter with its regulation has not been removed. Instead, the natural regulatory sequence has been mutated so that regulation no longer takes place and/or gene expression is increased. These modified promoters may also 25 be placed alone in the form of subsequences (= promoter with parts of the nucleic acid sequences according to the invention) in front of the natural gene to increase the activity. In addition, the gene construct may advantageously comprise one or more enhancer sequences functionally linked to the promoter, 30 which makes increased expression of the nucleic acid sequence possible. It is also possible to insert additional advantageous sequences at the 3' end of the DNA sequences, such as further regulatory elements or terminators. The  $\Delta 6$ -desaturase gene may be present in one or more copies in the expression cassette (= gene 35 construct). Any genes which are coexpressed and which are advantageously involved in fatty acid biosynthesis may also be present in the expression cassette in one or more copies.

The regulatory sequences or factors may, as described above, 40 preferably have a beneficial effect on the gene expression of the genes introduced, thus increasing it. Thus, enhancement of the regulatory elements can advantageously take place at the transcriptional level by using strong transcription signals such as promoters and/or enhancers. However, it is also possible to 45 enhance translation by, for example, improving the stability of mRNA.

Suitable promoters in the expression cassette are, in principle, all promoters which are capable of controlling the expression of foreign genes in organisms, advantageously in plants of fungi. It is preferable to use in particular a plant promoter or promoters derived from, for example, a plant virus. Examples of advantageous regulatory sequences for the process according to the invention are present, for example, in promoters such as the cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, lacI<sup>q</sup>, T7, T5, T3, gal, trc, ara, SP6, λ- $P_R$  or in the λ- $P_L$  promoter which are advantageously used in Gram-negative bacteria. Further advantageous regulatory sequences are present, for example, in the Gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, Mfα, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters such as CaMV/35S [Franck et al., Cell 15 21(1980) 285-294], RUBISCO SSU, OCS, B33, nos (= Nopaline Synthase Promoter) or in ubiquitin promoter. The expression cassette can also comprise a chemically inducible promoter by which expression of the exogenous Δ6-desaturase gene in the organisms, advantageously in the plants, can be controlled at a particular time. Examples of such advantageous plant promoters are the PRP1 promoter [Ward et al., Plant. Mol. Biol. 22 (1993), 361-366], a benzenesulfonamide-inducible promoter (EP 388186), a tetracycline-inducible promoter (Gatz et al., (1992) Plant J. 2,397-404), a salicylic acid-inducible promoter (WO 95/19443), an abscisic acid-inducible promoter (EP335528) and an ethanol- or cyclohexanone-inducible promoter (WO 93/21334). Further plant promoters are, for example, the potato cytosolic FBPase promoter, the potato ST-LSI promoter (Stockhaus et al., EMBO J. 8 (1989) 2445-245), the Glycine max phosphoribosyl-pyrophosphate amidotransferase promoter (see also Genbank Accession Number U87999) or a node-specific promoter in EP 249676 as can be advantageously used [sic]. Particularly advantageous plant promoters are those which ensure expression in tissues or plant parts/organs in which fatty acid biosynthesis or its precursors take place, such as, for example, in the endosperm or the developing embryo. Particular mention should be made of advantageous promoters which ensure seed-specific expression, such as, for example, the USP promoter or derivatives thereof, the LEB4 promoter, the phaseolin promoter or the napin promoter. The USP promoter which has been stated in accordance with the invention and which is particularly advantageous, or its derivatives, mediate very early gene expression during seed development (Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67). Other advantageous seed-specific promoters which can be used for monocotyledonous and dicotyledonous plants are the promoters suitable for dicots such as, for example, the oilseed rape napin gene promoter (US5,608,152), the *Arabidopsis* oleosin

promoter (WO98/45461), the *Phaseolus vulgaris* phaseolin promoter (US5,504,200), the *Brassica* Bce4 promoter (WO91/13980) or the legume B4 promoter (LeB4, Baeumlein et al., *Plant J.*, 2, 2, 1992: 233 – 239) or promoters which are suitable for monocots, such as 5 the barley lpt2- or lpt1-gene promoters (WO95/15389 and WO95/23230) or the promoters of the barley hordein gene, of the rice glutelin gene, of the rice oryzin gene, of the rice prolamин gene, of the wheat gliadin gene, of the wheat glutelin gene, of the maize zein gene, of the oat glutelin gene, of the sorghum 10 kasirin gene or of the rye secalin gene, which are described in WO99/16890.

Further particularly preferred promoters are those which ensure expression in tissues or plant parts in which, for example, the 15 biosynthesis of fatty acids, oils and lipids and their precursors takes place. Particular mention should be made of promoters which ensure seed-specific expression. Mention should be made of the oilseed rape napin gene promoter (US 5,608,152), the *Vicia faba* USP promoter (USP=unknown seed protein, Baeumlein et al., *Mol Gen 20 Genet*, 1991, 225 (3): 459-67), of the *Arabidopsis* oleosin gene (WO98/45461), of the phaseolin promoter (US 5,504,200) or of the legumin B4 gene promoter (LeB4; Baeumlein et al., 1992, *Plant Journal*, 2 (2): 233-9). Mention should furthermore be made of promoters such as that of the barley lpt2 or lpt1 gene 25 (WO95/15389 and WO95/23230), which ensure seed-specific expression in monocots.

The expression cassette (= gene construct, nucleic acid construct) may, as described above, comprise other genes which 30 are to be introduced into the organisms. These genes may be regulated separately or be in the same regulatory region as the  $\Delta 6$ -desaturase gene. These genes are advantageously further biosynthesis genes, advantageously of fatty acid biosynthesis, which allow increased synthesis. Examples which may be mentioned 35 are the genes for  $\Delta 15$ -,  $\Delta 12$ -,  $\Delta 9$ -,  $\Delta 5$ - and  $\Delta 4$ -desaturase, the various hydroxylases, the acyl ACP thioesterases,  $\beta$ -ketoacyl synthases or  $\beta$ -ketoacyl reductases. It is advantageous to use the desaturase genes in the nucleic acid construct.

40 In principle, it is possible for all natural promoters with their regulatory sequences like those mentioned above to be used for the expression cassette according to the invention and the process according to the invention, as described below. It is also possible and advantageous to use synthetic promoters.

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It is possible to manipulate various DNA fragments in order to obtain a nucleotide sequence which is expediently read in the

correct direction and which is equipped with a correct reading frame. To link the DNA fragments (= nucleic acids according to the invention) to each other adapters or linkers may be attached to the fragments.

5

Expediently, the promoter and terminator regions may be provided, in the direction of transcription, with a linker or polylinker comprising one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, in most cases 1 to 10 8, preferably 2 to 6, restriction sites. The size of the linker within the regulatory region is generally less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter can be either native, or homologous, or else foreign, or heterologous, in relation to the host organism, for example the 15 host plant. The expression cassette comprises in the 5'-3'-direction of transcription the promoter, a DNA sequence encoding a  $\Delta 6$ -desaturase gene used in the process according to the invention, and a region for transcriptional termination. Various termination regions can be exchanged for each other as 20 desired.

It is furthermore possible to employ manipulations which provide suitable restriction cleavage sites or which eliminate excess DNA or restriction cleavage sites. Where insertions, deletions or 25 substitutions such as, for example, transitions and transversions, are suitable, in vitro mutagenesis, primer repair, restriction or ligation may be used. In suitable manipulations such as, for example, restriction, chewing back or filling in overhangs for blunt ends, complementary ends of the fragments may 30 be provided for ligation.

Attachment of the specific ER retention signals SEKDEL (Schouten, A. et al., Plant Mol. Biol. 30 (1996), 781-792), may, inter alia, be of importance for advantageous high-level expression, thus 35 tripling to quadrupling the average level of expression. It is also possible to employ other retention signals which occur naturally with plant and animal proteins localized in the ER for constructing the cassette.

40 Preferred polyadenylation signals are plant polyadenylation signals, preferably those which correspond essentially to T-DNA-polyadenylation signals from *Agrobacterium tumefaciens*, in particular gene 3 of the T-DNA (octopin synthase) of the Ti-plasmids pTiACH5 (Gielen et al., EMBO J.3 (1984), 835 et seq.) 45 or corresponding functional equivalents.

DOCUMENT NUMBER

An expression cassette is generated by fusing a suitable promoter to a suitable  $\Delta 6$ -desaturase DNA sequence and to a polyadenylation signal by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. 5 Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, 10 Greene Publishing Assoc. and Wiley-Interscience (1987).

The DNA sequence encoding a *Phycomitrella* [sic] *patens*  $\Delta 6$ -desaturase comprises all sequence characteristics which are necessary to achieve correct localization for the site of fatty 15 acid, lipid or oil biosynthesis. No further targeting sequences are therefore necessary *per se*. However, such localization may be desirable and advantageous and can therefore be modified or enhanced artificially, so that such fusion constructs are also a preferred advantageous embodiment of the invention.

20 Particularly preferred sequences are those which ensure targeting into plastids. Under certain circumstances, targeting into other compartments (see review in: Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423) for example into the vacuole, into the 25 mitochondrion, into the endoplasmic reticulum (ER), peroxisomes, lipid bodies or, owing to the absence of suitable operative sequences, remaining in the compartment of formation, namely the cytosol, may also be desirable.

30 The nucleic acid sequences encoding  $\Delta 6$ -desaturase genes are advantageously cloned together with at least one reporter gene into an expression cassette which is introduced into the organism via a vector or directly into the genome. This reporter gene should make easy detection possible by a growth, fluorescence, 35 chemo- or bioluminescence or resistance assay or by a photometric measurement. Examples of reporter genes are genes for resistance to antibiotics or herbicides, hydrolase genes, fluorescence protein genes, bioluminescence genes, sugar or nucleotide metabolism genes or biosynthesis genes such as the *Ura3* gene, the 40 *Ilv2* gene, the luciferase gene, the  $\beta$ -galactosidase gene, the *gfp* gene, the 2-deoxyglucose-6-phosphate phosphatase gene, the  $\beta$ -glucuronidase gene,  $\beta$ -lactamase gene, the neomycin phosphotransferase gene, the hygromycin phosphotransferase gene or the BASTA (= gluphosinate [sic] resistance) gene. These genes 45 make it possible easily to measure and quantify the transcriptional activity and thus gene expression. It is thus possible to identify sites in the genome which show differences

in productivity.

In a preferred embodiment, an expression cassette comprises upstream, i.e. at the 5' end of the coding sequence, a promoter 5 and downstream i.e. at the 3' end, a polyadenylation signal, if appropriate, further regulatory elements which are operatively linked to the interposed coding sequence for the  $\Delta 6$ -desaturase DNA sequence. Operative linkage is to be understood as meaning the sequential arrangement of promoter, coding sequence, 10 terminator and, if appropriate, other regulatory elements in such a manner that each of the regulatory elements can carry out its function as intended in the expression of the coding sequence. The sequences preferred for operative linkage are targeting sequences for ensuring subcellular localization in plastids. 15 However, targeting sequences to ensure subcellular localization in the mitochondrion, in the endoplasmatic reticulum (ER), in the nucleus, in oleoplasts or other compartments may also be employed if required, as well as translation enhancers such as the tobacco mosaic virus 5' leader sequence (Gallie et al., Nucl. Acids Res. 20 15 (1987), 8693-8711).

An expression cassette can comprise, for example, a constitutive promoter (preferably the USP or napin promoter), the gene to be expressed and the ER retention signal. The ER retention signal, 25 which is preferably used is the amino acid sequence KDEL (lysine, aspartic acid, glutamic acid, leucine).

For expression in a prokaryotic or eukaryotic host organism, for example a microorganism such as a fungus or plant, the expression 30 cassette is advantageously inserted into a vector such as, for example, a plasmid, a phage or other DNA which allows optimal expression of the genes in the host organism. Examples of suitable plasmids are in *E. coli* pLG338, pACYC184, pBR series such as, for example, pBR322, pUC series such as pUC18 or pUC19, 35 M113mp series, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III<sup>113</sup>-B1,  $\lambda$ gt11 or pBdCI, in *Streptomyces* pIJ101, pIJ364, pIJ702 or pIJ361, in *Bacillus* pUB110, pC194 or pBD214, in *Corynebacterium* pSA77 or pAJ667, in fungi pALS1, pIL2 or pBB116, other advantageous fungal vectors being described by Romanos, 40 M.A. et al., [(1992) "Foreign gene expression in yeast: a review", *Yeast* 8: 423-488] and by van den Hondel, C.A.M.J.J. et al. [(1991) "Heterologous gene expression in filamentous fungi] and in *More Gene Manipulations in Fungi* [J.W. Bennet & L.L. Lasure, eds., pp. 396-428: Academic Press: San Diego] and in 45 "Gene transfer systems and vector development for filamentous fungi" [van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) in: *Applied Molecular Genetics of Fungi*, Peberdy, J.F. et al., eds.,

pp. 1-28, Cambridge University Press: Cambridge]. Advantageous yeast vectors are, for example 2 $\mu$ M, pAG-1, YEp6, YEp13 or pEMBLYe23. Examples of algal or plant promoters are pLGV23, pGHI<sup>lac</sup>, pBIN19, pAK2004, pVKH or pDH51 (see Schmidt, R. and Willmitzer, L., 1988). The abovementioned vectors or derivatives of the abovementioned vectors constitute a small selection of plasmids which are possible. Further plasmids are well known to the skilled worker and can be found, for example, in the book Cloning Vectors (Eds. Pouwels P.H. et al. Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018). Suitable plant vectors are described, inter alia, in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chap. 6/7, pp. 71-119. Advantageous vectors are shuttle vectors or binary vectors, which replicate in *E. coli* and *Agrobacterium*.

15

Apart from plasmids, vectors also mean all other vectors known to the skilled worker, such as, for example, phages, viruses such as SV40, CMV, baculovirus, adenovirus, transposons, IS elements, phasmids, phagemids, cosmids, linear or cyclic DNA. These vectors 20 are capable of autonomous replication or chromosomal replication in the host organism; chromosomal replication is preferred.

In a further embodiment of the vector, the expression cassette according to the invention can also advantageously be introduced 25 into the organisms in the form of a linear DNA and integrated into the genome of the host organism by heterologous or homologous recombination. This linear DNA may consist of a linearized plasmid or else only of the expression cassette as vector or the nucleic acid sequences according to the invention.

30

In a further advantageous embodiment, the nucleic acid sequence according to the invention can also be introduced alone into an organism.

35 If, in addition to the nucleic acid sequence according to the invention, further genes are to be introduced into the organism, it is possible to introduce them all together with a reporter gene in a single vector or each individual gene with a reporter gene in one vector in each case, or several genes together in 40 various vectors, into the organism, in which case the various vectors can be introduced simultaneously or successively.

The vector advantageously comprises at least one copy of the nucleic acid sequences encoding a  $\Delta 6$ -desaturase, and/or of the 45 expression cassette.

By way of example, the plant expression cassette can be incorporated into the transformation vector pRT ((a) Toepfer et al., 1993, *Methods Enzymol.*, 217: 66-78; (b) Toepfer et al. 1987, *Nucl. Acids. Res.* 15: 5890 et seq.).

5

As an alternative, a recombinant vector (= expression vector) can also be transcribed and translated in vitro, for example by using the T7 promoter and T7 RNA polymerase.

- 10 Expression vectors used in prokaryotes frequently make use of inducible systems with and without fusion proteins or fusion oligopeptides, it being possible for these fusions to take place both at the N terminus and at the C terminus or other domains of a protein which can be used. As a rule, such fusion vectors are 15 intended to: i.) increase the RNA expression rate, ii.) increase the protein synthesis rate which can be achieved, iii.) increase the solubility of a protein, or iv.) simplify purification by a binding sequence which can be used for affinity chromatography. Proteolytic cleavage sites are frequently also introduced by 20 fusion proteins, enabling elimination of part of the fusion protein also of the purification [sic]. Such recognition sequences for proteases recognize are [sic], for example, factor Xa, thrombin and enterokinase.
- 25 Typical advantageous fusion and expression vectors are pGEX [Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67: 31-40], pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ), which comprises glutathione S transferase (GST), maltose binding protein, or protein A.

30

- Further examples of *E. coli* expression vectors are pTrc [Amann et al., (1988) *Gene* 69:301-315] and pET vectors [Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89; Stratagene, Amsterdam, 35 The Netherlands].

- Further advantageous vectors for use in yeasts are pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., 40 (1987) *Gene* 54:113-123), and pYES derivatives (Invitrogen Corporation, San Diego, CA). Vectors for use in filamentous fungi are described in: van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: *Applied Molecular Genetics of Fungi*, J.F. Peberdy, et 45 al., eds., pp. 1-28, Cambridge University Press: Cambridge.

As an alternative, insect cell expression vectors may also be used advantageously, for example for the expression in Sf 9 cells. Examples of these are the vectors of the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and of the pVL series 5 (Lucklow and Summers (1989) *Virology* 170:31-39).

Moreover, plant cells or algal cells may advantageously be used for gene expression. Examples of plant expression vectors are found in Becker, D., et al. (1992) "New plant binary vectors with 10 selectable markers located proximal to the left border", *Plant Mol. Biol.* 20: 1195-1197 or in Bevan, M.W. (1984) "Binary *Agrobacterium* vectors for plant transformation", *Nucl. Acid. Res.* 12: 8711-8721.

15 Moreover, the nucleic acid sequences encoding Δ6-desaturase may also be expressed in mammalian cells. Examples of suitable expression vectors are pCDM8 and pMT2PC, mentioned in: Seed, B. (1987) *Nature* 329:840 or Kaufman et al. (1987) *EMBO J.* 6: 187-195). Promoters preferably to be used in such cases are of 20 viral origin, such as, for example, promoters of polyoma virus, adenovirus 2, cytomegalovirus or simian virus 40. Further prokaryotic and eukaryotic expression systems are mentioned in Chapters 16 and 17 in Sambrook et al., *Molecular Cloning: A Laboratory Manual*. 2nd ed., Cold Spring Harbor Laboratory, Cold 25 Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

The introduction of the nucleic acids according to the invention, of the expression cassette or of the vector into organisms, for example into plants, can in principle take place by all methods 30 known to the skilled worker.

The skilled worker can find suitable methods for microorganisms in the textbooks by Sambrook, J. et al. (1989) *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, by F.M. 35 Ausubel et al. (1994) *Current protocols in molecular biology*, John Wiley and Sons, by D.M. Glover et al., *DNA Cloning Vol.1*, (1995), IRL Press (ISBN 019-963476-9), by Kaiser et al. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press or Guthrie et al. *Guide to Yeast Genetics and Molecular Biology*, 40 *Methods in Enzymology*, 1994, Academic Press.

The transfer of foreign genes into the genome of a plant is termed transformation. Use is made here of the above-described methods for the transformation and regeneration of plants from 45 plant tissues or plant cells for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic method with

the gene cannon, the particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and agrobacterium-mediated gene transfer. The methods mentioned are described, for example, by B. 5 Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993) 128-143 and Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991) 205-225). The construct to be expressed advantageously clones into a vector 10 suitable for transforming *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711). *Agrobacteria* transformed with such a vector can then be used in the known manner for transforming plants, in particular crop plants such as, for example tobacco plants, for example by 15 bathing scarified leaves or leaf sections in an agrobacterial solution and subsequently growing them in suitable media. The transformation of plants with *Agrobacterium tumefaciens* is described, for example, by Höfgen and Willmitzer in Nucl. Acid Res. (1988) 16, 9877 or is known, inter alia, from F.F. White, 20 Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38.

*Agrobacteria* transformed with an expression vector as described 25 above can also be used in a known manner for transforming plants such as test plants such as *Arabidopsis* or crop plants such as cereals, maize, oats, rye, barley, wheat, soybean, rice, cotton, sugarbeet, canola, triticale, sunflower, flax, hemp, potato, tobacco, tomato, coffee, cacao, tea, carrot, capsicum, oilseed 30 rape, tapioca, carcaba, arrowroot, tagetes, alfalfa, lettuce and the various tree, nut and grapevine species, in particular oil-containing crop plants such as soybean, peanut, castor, borage, linseed, sunflower, canola, cotton, flax, oilseed rape, coconut, oilpalm, safflower (*Carthamus tinctorius*) or cacao bean, 35 for example by bathing scarified leaves or leaf sections in an agrobacterial solution and subsequently growing them in suitable media.

The genetically modified plant cells can be regenerated by all 40 methods known to the skilled worker. Suitable methods can be found in the abovementioned publications by S.D. Kung and R. Wu, Potrykus or Höfgen and Willmitzer.

Organisms or host organisms for the nucleic acids used [lacuna] 45 processes according to the invention, the expression cassette used or the vector used are, in principle and advantageously, all organisms which are capable of synthesizing fatty acids;

specifically unsaturated fatty acids, or which are suitable for the expression of recombinant genes. Examples which may be mentioned are plants such as *Arabidopsis*, *Asteraceae* such as *calendula*, or crop plants such as soybean, peanut, castor, 5 sunflower, maize, cotton, flax, oilseed rape, coconut, oilpalm, safflower (*Carthamus tinctorius*) or cacao bean, microorganisms such as fungi, for example the genus *Mortierella*, *Saprolegnia* or *Pythium*, bacteria such as the genus *Escherichia*, cyanobacteria, ciliates, *thraustochytria* or *schizichytria*, algae or protozoa 10 such as dinoflagellates such as *Cryptocodium*. Preferred organisms are those which are capable of naturally synthesizing oils in substantial amounts, such as fungi of the genera *Mortierella* or *Pythium*, such as *Mortierella alpina* and *Pythium insidiosum*, or plants such as soybean, oilseed rape, coconut, 15 oilpalm, safflower, castor, *calendula*, peanut, cacao bean or sunflower, with soybean, oilseed rape, sunflower, castor, *Mortierella* or *Pythium* being especially preferred. In principle, transgenic animals, for example *C. elegans*, are also suitable as host organisms.

20 Host cells which can be used are also mentioned in: Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990).

25 Expression strands which can be used, for example those which have a lower protease activity, are described in: Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128.

30 Depending on the choice of the promoter, expression of the  $\Delta 6$ -desaturase gene may take place specifically in the leaves, in the seeds, the tubers or other parts of the plant. The present invention furthermore relates to such transgenic plants which overproduce fatty acids, oils or lipids, and to their propagation 35 material and their plant cells, tissue or plant parts. A preferred subject according to the invention is transgenic

plants, for example crop plants such as maize, oats, rye, wheat, barley, maize [sic] rice, soybean, sugarbeet, canola, triticale, sunflower, flax, hemp, tobacco, tomato, coffee, cacao, tea, 40 carrot, capsicum, oilseed rape, tapioca, carica, arrowroot, tagetes, alfalfa, lettuce and the various tree, nut and grapevine species, potatoes, in particular oil-containing crop plants such as soybean, peanut, castor, borage, linseed, sunflower, canola, cotton, flax, oilseed rape, coconut, oilpalm, safflower 45 (*Carthamus tinctorius*) or cacao bean, laboratory plants such as *Arabidopsis*, or other plants such as mosses or algae comprising a functional nucleic acid sequence according to the invention or a

functional expression cassette. Functional in this context means that an enzymatically active enzyme is formed.

The expression cassette or the nucleic acid sequences according 5 to the invention comprising a  $\Delta 6$ -desaturase gene sequence can additionally also be used for the transformation of the organisms which have been mentioned above by way of example, such as bacteria, cyanobacteria, filamentous fungi, ciliates, animals or algae, with the aim of increasing the content in fatty acids, 10 oils or lipids [lacuna] of  $\Delta 6$ -double bonds. Preferred transgenic organisms are bacteria, cyanobacteria, filamentous fungi or algae.

Transgenic organisms are to be understood as meaning organisms 15 which comprise a foreign nucleic acid derived from another organism which encodes a  $\Delta 6$ -desaturase used in the process according to the invention. Transgenic organisms are also to be understood as meaning organisms which comprises [sic] a nucleic acid which is derived from the same organism and encodes a 20  $\Delta 6$ -desaturase, this nucleic acid being present as an additional gene copy or not being present in the natural nucleic acid environment of the  $\Delta 6$ -desaturase gene. Transgenic organisms are also organisms in which the natural 3'- and/or 5'-region of the  $\Delta 6$ -desaturase gene has been modified over the initial organisms 25 by targeted, recombinant modifications. Preferred transgenic organisms are those into which a foreign DNA has been introduced. Especially preferred are transgenic plants into which a foreign DNA has been introduced. Transgenic plants are to be understood as meaning individual plant cells and their cultures, such as, 30 for example, callus cultures on solid media or in liquid culture, the plant parts and intact plants.

The invention furthermore relates to transgenic organisms selected from the group of the plants, fungi, ciliates, algae, 35 bacteria, cyanobacteria or animals, preferably transgenic plants or algae, comprising at least one isolated nucleic acid sequence encoding a polypeptide with  $\Delta 6$ -desaturase activity, selected from the group consisting of:

- 40 a) a nucleic acid sequence having the sequence shown in SEQ ID NO: 1,
- b) nucleic acid sequences which, as a result of the degeneracy of the genetic code, are derived from [lacuna] shown in SEQ 45 ID NO: 1,

- c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1 which encode polypeptides with the amino acid sequences shown in SEQ ID NO: 2 and have at least 50% homology at the amino acid level without substantially reducing the enzymatic 5 action of the polypeptides.

Increasing the content of fatty acids, oils or lipids with  $\Delta 6$ -double bonds means for the purposes of the present invention for example the artificially acquired ability of an increased 10 biosynthesis performance by functionally overexpressing the  $\Delta 6$ -desaturase gene in the organisms according to the invention, advantageously in the transgenic plants according to the invention, in relation to the nonrecombinant initial plants, at least for the duration of at least one plant generation.

15 The biosynthesis site of fatty acids, oils or lipids, for example, is generally the seed or cell layers of the seed, so that seed-specific expression of the  $\Delta 6$ -desaturase gene is meaningful. However, it is obvious that the biosynthesis of fatty 20 acids, oils or lipids need not be restricted to the seed tissue, but may also take place in a tissue-specific manner in all remaining parts of the plant, for example in epidermis cells or in the tubers.

25 In addition, constitutive expression of the exogenous  $\Delta 6$ -desaturase gene is advantageous. However, inducible expression may also be desirable.

The efficacy of expression of the  $\Delta 6$ -desaturase gene can be 30 determined for example in vitro by shoot meristem propagation. In addition, an expression of the  $\Delta 6$ -desaturase gene whose type and level has been modified, and its effect on fatty acid, oil or lipid biosynthetic activity can be tested in glasshouse experiments on test plants.

35 The invention relates to transgenic plants as described above, transformed with a nucleic acid sequence encoding a  $\Delta 6$ -desaturase, a vector or an expression cassette comprising a  $\Delta 6$ -desaturase gene sequence or DNA sequences hybridizing herewith, 40 and to transgenic cells, tissue, parts and propagation material of such plants. Especially preferred in this context are transgenic crop plants as described above.

Plants for the purposes of the invention are monocots and dicots 45 or algae.

The invention furthermore relates to:

- the use of a  $\Delta 6$ -desaturase DNA gene sequence with the sequence stated in SEQ ID NO:1 or DNA sequences hybridizing herewith for the generation of fungi, bacteria, animals or plants, preferably plants, with an increased content of fatty acids, oils or lipids with  $\Delta 6$ -double bonds by expressing this  $\Delta 6$ -desaturase DNA sequence in plants.
- 5 10 - the use of the proteins with the sequences SEQ ID NO: 2 for the preparation of unsaturated fatty acids in plants, fungi, bacteria or animals, preferably plants.

The invention is illustrated in greater detail by the examples 15 which follow:

#### Examples

##### Example 1: General cloning and culture methods:

20 The cloning methods such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking of DNA-fragments, transformation of *Escherichia coli* 25 cells, cultivation of organisms, and the sequence analysis of recombinant DNA, were carried out as described by Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6). The protonema of *Physcomitrella patens* (= *P. patens*) was cultured in liquid medium as described by Reski et al. (Mol. Gen. Genet., 30 244, 1994: 352-359).

##### Example 2: Recombinant DNA sequence analysis:

35 Recombinant DNA molecules were sequenced using an ABI laser fluorescence DNA sequencer by the method of Sanger (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA74, 5463-5467). Fragments resulting from a polymerase chain reaction were sequenced and checked to avoid polymerase errors in constructs to be expressed.

40 Example 3: Analysis of lipid from the *P. patens* Protonema and from yeast cells

The lipids were extracted from the *S. [sic] patens* Protonema or 45 from yeast cells using chloroform/methanol as described by Siebertz et al. (Eur. J. Biochem., 101, 1979: 429-438) and purified with diethyl ether by thin-layer chromatography (= TLC). The fatty acids obtained were transmethylated to give the

corresponding methyl esters and analyzed by gas chromatography (= GC). The various methyl esters were identified using corresponding standards. Corresponding fatty acid pyrrolidides were obtained, and identified by GC-MS, as described by Anderson et al. (Lipids, 9, 1974: 185-190).

Example 4: Functional expression of the *P. patens*  $\Delta 6$ -desaturase cDNA in yeasts

10 The expression experiments in yeasts were carried out with PPDES6 cDNA. Knock-out experiments had shown (data and experimental procedure not shown or described) that the knock-out effect leads to a loss of 20:3<sup>11,14,17</sup>-, 20:4<sup>5,8,11,14</sup>-, 20:4<sup>5,11,14,17</sup>- and 20:5<sup>5,8,11,14,17</sup>-fatty acids. The 18:2<sup>9,12</sup>- and 18:3<sup>9,12,15</sup>-fatty acids increase simultaneously. For expression in yeast, the PPDES6 cDNA was subcloned into the yeast expression vector pYES2 (Invitrogen). The vector obtained was named pYESdelta6. Yeast cultures transformed with pYES2 (control) and pYESdelta6 ( $\Delta 6$ -desaturase cDNA) were cultured on uracil drop-out medium 20 supplemented with 2% raffinose and 1% Tergitol NP-40 (for stabilizing the fatty acids). For expression, the cells were cultured with galactose (final concentration 2%) up to an optical density (= OD) of 0.5 and 600 nm. In feeding experiments, fatty acids were solubilized in 5% Tergitol and added at a final 25 concentration of 0.0003%. The results of expression can be found in Table I. The synthesis of fatty acids with a double bond at position 6 is only possible in the presence of the expression construct with the  $\Delta 6$ -desaturase cDNA. This  $\Delta 6$ -desaturase enzyme had a greater activity in relation to fatty acids which already 30 contain a double bond at position 9 or 12 (relative to carbon atom in the chain). The fatty acid methyl esters of all of the yeast lipids were analyzed by GC. The individual fatty acids synthesized are shown in the table in mol% of the overall fatty acids.

35

40

45

Table I: Fatty acid composition in transformed yeasts in relation to the control

Overall fatty acids (%)				
	pYES2		pYESdelta6	
Fatty acids	—	—	+ 18:2 <sup>9,12</sup>	+ 18:3 <sup>9,12,15</sup>
16:0	16.4	16.1	23.8	25.8
16:1 <sup>9</sup>	54.0	55.5	38.1	31.4
16:2 <sup>6,9</sup>	—	4.2	1.7	—
18:0	3.2	2.4	4.0	—
18:1 <sup>9</sup>	24.9	19.7	19.1	19.2
18:2 <sup>6,9</sup>	—	0.6	0.2	—
18:2 <sup>9,12</sup>	—	—	8.5	—
18:3 <sup>6,9,12</sup>	—	—	4.0	—
18:3 <sup>9,12,15</sup>	—	—	—	11.7
18:4 <sup>6,9,12,15</sup>	—	—	—	3.0

20 Example 5: Transformation of *P. patens*

The polyethylene glycol-mediated direct DNA transformation of protoplasts was carried out as described by Schäfer et al. (Mol. Gen. Genet., 226, 1991: 418–424). The transformants were selected on G418-containing medium (Girke et al., The Plant Journal, 15, 1998: 39–48).

30 Example 6: Isolation of Δ6-desaturase cDNA and genomic clones of *P. patens*

Eventually fragments of a Δ6-desaturase gene were cloned with the aid of a PCR reaction with the following degenerate oligonucleotides as primers:

35 A: TGGTGGAA(A/G)TGGA(C/A)ICA(T/C)AA and  
 B: GG(A/G)AA(A/C/G/T)A(A/G)(G/A)TG(G/A)TG(C/T)TC]

and the following temperature program:

40 94°C, 3 min; [94°C, 20 sec; 45°C, 30 sec; 72°C, 1 min], 30 cycles; 72°C, 5 min. For cloning, poly(A)RNA was isolated from 12-day-old *P. patens* Protonema culture [sic]. The above-described PCR was carried out with this poly(A)RNA. Fragments of the expected fragment length (500 to 600 bp) were cloned into pUC18 and 45 sequenced. The deduced amino acid sequence of a PCR fragment showed similarities with known Δ6-desaturases. Since it was known that *P. patens* has a Δ6-desaturase, it was assumed that this clone

encodes part of a  $\Delta 6$ -desaturase.

A complete cDNA clone (= PPDES6 cDNA) was isolated from *P. patens* cDNA library of 12-day-old Protonemata with the aid of the PCR 5 fragment specified above. The nucleotide sequence is shown in SEQ ID NO:1. The deduced amino acid sequence can be seen from SEQ ID NO:2. The corresponding genomic sequence (= PPDES6 gene) was isolated with the aid of the PCR and the following oligonucleotides as primers:

10

C: CCGAGTCGCGGATCAGCC  
D: CAGTACATTGGTCATTCACC:

Table II shows the results of the comparison between the novel *P. patens*  $\Delta 6$ -desaturase over the entire nucleic acid sequence with 15 the following, known  $\Delta 6$ -desaturase: *Borago officinalis* (U79010), *Synechocystis* sp (L11421), *Spirulina platensis* (X87094), *Caenorhabditis elegans* (AF031477), *Mortierella alpina* (WO 98/46764), *Homo sapiens* (Cho et al., *J. Biol. Chem.*, 274, 1999: 20 471-477), *Rattus norvegicus* (AB021980) and *Mus musculus* (Cho et al., *J. Biol. Chem.*, 274, 1999: 471-477). The analysis was carried out with the Gap Program (GCG Package, Version 9.1) and the following analysis parameters: scoring matrix, blosum62, gap creation penalty, 12; gap extension penalty, 4. The results show 25 the particular identity or similarity [] in percent (%) in relation with the *P. patens* sequence.

Table II: Sequence comparison between *P. patens*  $\Delta 6$ -desaturase and other  $\Delta 6$ -desaturases

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Sequence	Amino acid sequence identity [similarity] (%)
<i>Borago officinalis</i>	31 [38]
<i>Synechocystis</i> sp.	21 [29]
<i>Spirulina platensis</i>	20 [29]
<i>Caenorhabditis elegans</i>	35 [43]
<i>Mortierella alpina</i>	39 [47]
<i>Homo sapiens</i>	27 [38]
<i>Rattus norvegicus</i>	28 [39]
<i>Mus musculus</i>	29 [39]

Example 7: Cloning the *Physcomitrella patens*  $\Delta 6$ -desaturase

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The genomic  $\Delta 6$ -acyllipid desaturase from *Physcomitrella patens* was modified, isolated and used in the process according to the

invention on the basis of the published sequence (Girke et al., Plant J., 15, 1998: 39-48) using a polymerase chain reaction and cloning. To this end, a desaturase fragment was first isolated by means of polymerase chain reaction using two gene-specific 5 primers, and inserted into the desaturase gene described in Girke et al. (see above).

Primer TG5: 5'-ccgctcgagcgagggtgtggagcggc and  
Primer TG3: 5'-ctgaaatagtcttgctcc-3'

10 were first used for amplifying a gene fragment by means of polymerase chain reaction (30 cycles, 30 sec. at 94°C [sic], 30 sec. at 50°C, 60 sec. at 72°C, post-incubation for 10 minutes at 72°C, in a Perkin Elmer thermocycler).

15 a) Cloning an expression plasmid expressing Δ6-desaturase under the control of the 35S CaMV [sic] promoter:

20 An XhoI cleavage site was introduced into the fragment by the primer TG5. An XhoI/Eco47III fragment was obtained by restriction and transposed into the PPDES6 gene sequence described in Girke et al. following analogous restriction with XhoI/Eco47III. The construct was named pZK. The insert of pZK was cloned into the XhoI/SmaI cleavage site of 25 pRT99/35S as XhoI/HindIII fragment after filling up the HindIII cleavage site with nucleotides by treatment with the Klenow fragment of DNA polymerase I. The resulting plasmid pSK contains the 35S promoter [cauliflower mosaic virus, Franck et al. (1980) Cell 21, 285], the moss Δ6-desaturase 30 and the 35S terminator in the vector pRT.

b) Construction of an expression construct under the control of the napin promoter:

35 The resulting promoter desaturase fragment with terminator was cloned into the vector pJH3 by cleaving the plasmid pSK with XhoI, treatment with T4 DNA polymerase and PstI restriction. To this end, the vector BamHI was cleaved, the overhangs were filled up with Klenow enzyme, and this was 40 followed by cutting with PstI. Ligation of the desaturase terminator fragment into the vector gave rise to the plasmid pJH7, which contains a napin promoter (Scofield et al., 1987, J. Biol. Chem. 262, 12202-8). The expression cassette of pJH7 was cleaved with Bsp120I and NotI and cloned into the binary 45 vector pRE. This gave rise to the plasmid pRE-Ppdes6.

In a PCR reaction, the *P. patens*  $\Delta 6$ -desaturase cDNA according to the invention was used as template. With the aid of the oligonucleotides stated hereinbelow, a BamHI restriction cleavage site was introduced before the start codon and three adenine nucleotides were introduced into the  $\Delta 6$ -desaturase cDNA as consensus translation sequence for eukaryotes. A 1512 base pair fragment of the  $\Delta 6$ -desaturase was amplified and sequenced.

10 Pp-d6Des1: 5'- CC GGTACC aaaatggtattcgcgccggcggtg -3'  
 Pp-d6Des2: 3'- CC GGTACC ttaactggtggtacatgct -3'

15 The reaction mixtures contained approximately 1 ng/micro l [sic] template DNA, 0.5  $\mu$ m of the oligonucleotides and, 200  $\mu$ m deoxy-nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM MgCl<sub>2</sub>) and 0.02 U/ $\mu$ l Pwo polymerase (Boehringer Mannheim) and are incubated in a Perkin Elmer PCR machine with the following temperature program:

20 Annealing temperature: 50°C, 30 sec  
 Denaturation temperature: 95°C, 30 sec  
 Elongation temperature: 72°C, 90 sec  
 Number of cycles: 30

25 c) Construction of an expression construct under the control of the USP promoter:  
 The resulting fragment of approx. 1.5 kB base pairs was ligated into the vector pBluescript SK- (Stratagene) which 30 had been cleaved with EcoRV and was available for further clonings as BamHI fragment.

35 For the transformation of plants, a further transformation vector based on pBin-USP was generated, and this transformation vector contains the  $\Delta 6$ -desaturase BamHI fragment. pBin-USP is a derivative of plasmid pBin19. pBinUSP originated from pBin19, by inserting an USP promoter into pBin19 [Bevan et al. (1980) Nucl. Acids Res. 12, 8711] as EcoRI-BamHI [sic] fragment. The polyadenylation signal is 40 that of gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al., (1984) EMBO J. 3, 835), where the nucleotides 11749-11939 were isolated as PvuII-HindIII fragment and, after the addition of SphI-linkers, cloned at the PvuII 45 cleavage site between the SphI-HindIII [sic] cleavage site of the vector. The USP promoter corresponds to the nucleotides 1-684 (Genbank Accession X56240), where part of the noncoding region of the USP gene was obtained in the promoter. The

promoter fragment which is 684 base pairs in size was amplified with the aid of commercially available T7 standard primer (Stratagene) and with the aid of a synthesized primer via a PCR reaction using standard methods (primer sequence: 5'-GTCGACCCGCGGACTAGTGGGCCCTAGACCCGGGGATCC GGATCTGCTGGCTATGAA-3'). The PCR fragment was subsequently cut with EcoRI/SalI and inserted into the vector pBin19 with OCS terminator. This gave rise to the plasmid named pBinUSP.

- 10 d) Construction of an expression construct under the control of the Beta vulgaris vATPase C1 promoter:

A construct using the v-ATPase c1 promoter was generated analogously to the expression plasmid with the USP promoter.

- 15 The promoter was cloned into the plasmid pBin19 with OCS terminator as an EcoRI/KpnI fragment and the *P. patens*  $\Delta$ 6-desaturase gene was inserted between promoter and terminator via BaMHI. The promoter corresponds to a beta [sic] *Vulgaris* [sic] 1153 base pair fragment (Plant Mol Biol, 20 1999, 39:463-475).

The construct was employed for the transformation of *Arabidopsis thaliana* and oilseed rape plants.

- 25 Example 8: Generation of transgenic oilseed rape plants (modified according to Moloney et al., 1992, Plant Cell Reports, 8:238-242)

To generate transgenic oilseed rape plants, binary vectors were 30 made use of in *Agrobacterium tumefaciens* C58C1:pGV2260 or *Escherichia coli* (Deblaere et al, 1984, Nucl. Acids. Res. 13, 4777-4788). To transform oilseed rape plants (var. Drakkar, NPZ Norddeutsche Pflanzenzucht, Hohenlieth, Germany), a 1:50 dilution of an overnight culture of a positively transformed agrobacterial 35 colony grown in Murashige-Skoog Medium (Murashige and Skoog 1962 Physiol. Plant. 15, 473) supplemented with 3% of sucrose (3MS medium) was used. Petioles or hypocotyledons [sic] of freshly germinated sterile oilseed rape plants (in each case approx. 1 cm<sup>2</sup>) were incubated for 5-10 minutes in a Petri dish together with 40 a 1:50 agrobacterial dilution. This was followed by 3 days' incubation in the dark at 25°C on 3MS medium with 0.8% Bacto agar. After 3 days, the culture was continued under 16 hours light/8 hours dark, and continued in a weekly rhythm on MS medium 45 supplemented with 500 mg/l Claforan (cefotaxime sodium), 50 mg/l kanamycin, 20  $\mu$ M benzylaminopurine (BAP) and 1.6 g/l glucose. Growing shoots were transferred to MS medium supplemented with sucrose, 250 mg/l Claforan and 0.8% Bacto agar. If no roots have

formed after three weeks, 2-indolebutyric acid was added to the medium as growth hormone for rooting.

Regenerated shoots were obtained on 2MS medium supplemented with 5 kanamycin and Claforan, then, after rooting, transferred into soil and, after cultivation for two weeks, grown in a controlled-environment cabinet or in the greenhouse and allowed to flower, and mature seeds were harvested and analyzed for  $\Delta 6$ -desaturase expression by means of lipid analyses. Lines with 10 increased contents of or [sic] double bonds at the  $\Delta 6$  position were identified. In the stably transformed transgenic lines which functionally expressed the transgene, an increased content of double bonds at position  $\Delta 6$  was found in comparison with untransformed control plants.

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Example 8 [sic]: Lipid extraction from seeds

The plant material was first homogenized mechanically by comminuting in a pestle and mortar to make it more accessible to 20 extraction.

Then, it was boiled for 10 minutes at 100°C and sedimented after cooling on ice. The cell sediment was hydrolyzed for one hour at 90°C with 1 N of methanolic sulfuric acid and 2% dimethoxypropane 25 and the lipids were transmethylated. The resulting fatty acid methyl esters (FAMEs) were extracted in petroleum ether. The extracted FAMEs were analyzed by gas liquid chromatography using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0.32 mm) and a temperature gradient of 170°C to 240°C over 30 20 minutes and 5 minutes at 240°C. The identity of the fatty acid methyl esters was confirmed by comparison with corresponding FAME standards (sigma). The identity and position of the double bond could be analyzed further by suitable chemical derivatization of the FAME mixtures, for example to give 35 4,4-dimethoxyxazoline derivatives (Christie, 1997, in: Advances in Lipid Methodology, 4<sup>th</sup> Edition: Christie, Oily Press, Dundee, 119–169, and 1998, Gaschromatographie–Massenspektrometrie Verfahren [Gas chromatography/mass spectrometry methods], Lipide 33:343–353) using GC-MS. The GC analysis of the fatty acid methyl 40 esters from the transgenic rapeseed which expressed  $\Delta 6$ -desaturase in a seed-specific fashion are shown in Table III. The transgenic rapeseed shows at least 4.95%  $\gamma$ -linolenic acid in the seed.

Table III shows the GC analyses of the fatty acid methyl esters 45 from mature, transgenic rapeseed which expressed  $\Delta 6$ -desaturase in a seed-specific fashion. The fatty acid composition is shown in [mol%] of the overall fatty acids. It can be stated that

individual plants of the T2 generation which have been obtained from positively transformed, selfed plants contain up to approx. 4.95% of  $\gamma$ -linolenic acid.

5 Table III: GC analysis of the oilseed rape fatty acid methyl esters

	Name	18:0	18:1	18:2	18:3( $\gamma$ )	18:3( $\alpha$ )	18:4
10	R2-T2-11/1a	1.98	53.58	22.63	3.86	11.38	0
	R2-T2-11/1b	1.86	52.04	25.45	2.31	11.39	0
	R2-T2-11/1c	1.95	49.17	24.30	2.84	9.20	0
	R2-T2-11/3	1.82	49.83	24.54	3.88	10.12	0
	R2-T2-11/4	1.72	48.02	24.66	4.95	9.52	0
	R2-T2-11/5a	1.73	51.98	25.27	4.27	9.61	0
	R2-T2-11/5b	2.02	56.19	25.08	0	9.33	0
	R2-T2-11/5c	2.01	46.95	27.38	0	10.37	0
	R2-T2-11/5d	1.83	49.49	24.15	4.40	8.65	0
	R2-T2-11/6	2.08	54.52	23.94	2.05	9.29	0
15	R2-T2-11/10	1.94	53.92	22.81	4.06	9.44	0
	R2-T2-WT	1.90	47.75	30.91	0	10.51	0
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